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TITLE: Study the Origin and Mechanisms of Castration Resistance Characterized by Outgrowth of Prostate Cancer Cells with Low/Negative Androgen Receptor

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14. ABSTRACT

Prostate cancer is the most common form of cancer in men with highly variable clinical response, reflecting the heterogeneity of the disease. Indeed, several recent studies have highlighted the molecular and genetic diversity among prostate cancers including a wide range of androgen receptor (AR) activity, which is the central axis of the disease. Interestingly AR activity did not show high correlation with AR mRNA or protein level in tumors, implicating other factors in this phenomenon. Given that AR is the central therapeutic target in this disease, we asked if AR targeted therapy have different impact on tumors with differential AR activity. Our data provided evidence that prostate cancer cells with varying AR activities have different molecular characteristics and the tumors with high AR activity are more resistant to AR-targeted therapy. We also identified GREB1 as a potential new AR cofactor that enhances AR activity and Enzalutamide resistance in tumors with high AR activity. Further understanding of the function of GREB1 will provide novel insights into the development of effective therapeutic approaches to treat Enzalutamide resistant prostate cancer.

15. SUBJECT TERMS

Study the molecular characteristics and Enzalutamide sensitivity of prostate cancer with different AR activity.

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Introduction

Prostate cancer is the most common form of cancer in men. Given the key role of androgen receptor (AR) signaling in disease progression, the current approach to treat prostate cancer is AR-targeted therapy. While this initially results in tumor regression, aggressive disease typically recurs, making the treatment of what is now called castration-resistant prostate cancer (CRPC) the major challenge in the field (1). In CRPC, conventional AR therapy fails to block AR activity with diverse mechanisms (2, 3), reflecting the heterogeneity of the disease. Indeed, several recent studies have highlighted the molecular and genetic diversity among prostate cancers (4, 5) including a wide range of AR activity (6). Interestingly AR activity did not show high correlation with AR mRNA or protein level in tumors (6), implicating other factors in this phenomenon. Given that AR is the central therapeutic target in this disease, we first asked if AR targeted therapy have different impact on tumors with differential AR activity, and also studied the underlying mechanisms of heterogeneous AR activity in prostate cancer

Keywords

Prostate cancer; Androgen receptor; Castration-resistant prostate cancer (CRPC); Enzalutamide resistance; GREB1; p300

Accomplishments

Specific Aim 1: Determine if prostate cancer cells with different AR activities have different response to AR-targeted therapy.

1-1. Determine if the different AR activities within prostate cancer cells are stable phenotype.

Proposed completion date: 09.2016-05.2016 (9 months)

Actual completion date: 09.2016-02.2016 (6 months)

Percentage of completion: 100%

Results: To isolate cells with different AR activities, we stably transduced prostate cancer cells with an eGFP AR reporter. Consistent with the clinical data, we observed wide range of AR activity when the GFP level was analysed by flow cytometry (Fig1A). To capture cells with low (AR-low) and high (AR-hi) AR activity, we sorted out 5% of the entire population with lowest and highest GFP expression, respectively (Fig1A). When we compared AR and AR target gene levels in sorted AR-low and -hi cells, AR-hi cells have higher AR target gene expression than AR-low cells (Fig1B), indicating that the reporter construct used in this study well reflects the endogenous AR activity. Surprisingly, the two populations have comparable AR expression shown by both mRNA and protein levels (Fig1B), suggesting that there are other factors than AR itself causing different AR output in these populations. The sorted AR-low and –hi cells maintained their AR activities over 30 days (Fig1C), suggesting the two cell populations might have different molecular characteristics. Furthermore, AR-hi cells showed enhanced up-regulation of AR target genes in response to DHT treatment (Fig1D), suggesting that AR-low and AR-hi cells have differential AR transcriptional activity.

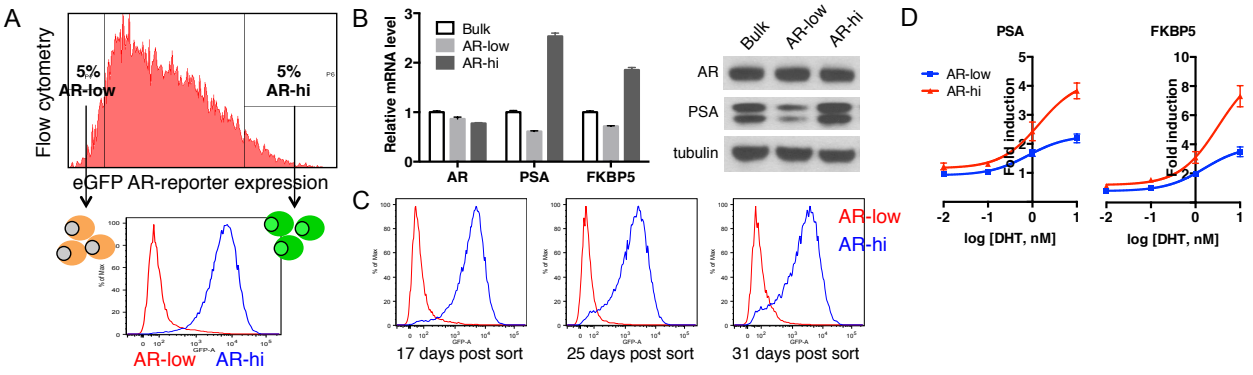

Figure 1. Characterization of prostate cancer cells with low vs. high AR activity. (A) LNCaP cells with low (AR-low) and high (AR-hi) AR activity were sorted out using flow cytometry based on eGFP AR-reporter expression. (B) AR-hi cells have higher AR output while having same level of AR. (C) AR-low and AR-hi cells maintain their AR activity over time. (D) AR-hi cells have enhanced DHT-induced AR transcriptional activity compared to AR-low cells.

1-2. Compare Enzalutamide sensitivity between cells with low vs high AR activities.

Proposed completion date: 03.2016-08.2016 (6 months)

Actual completion date: 03.2016-08.2016 (6 months)

Percentage of completion: 100%

Results: Next we tested if the two populations have different sensitivity to Enzalutamide by culturing the sorted cells with/without Enzalutamide. Interestingly, the in vitro growth assay shows that AR-low cells grow faster than bulk and AR-hi populations in vehicle treated condition (Fig2A). Furthermore, contrary to our prediction that AR-low cells will be less dependent on AR activity, Fig2A shows that AR-low cells are in fact more sensitive to Enzalutamide than AR-hi cells. Consistent with the in vitro results, tumors derived from AR-low population showed enhanced growth when injected into SCID mice (Fig2B). When the sorted populations were injected into castrated SCID mice and treated with Enzalutamide immediately after injection, tumors from AR-high cells were more resistant to this treatment than other populations (Fig2D). At the end of the experiments we examined AR activity of the tumors by analyzing the activity of GFP AR reporter in individual tumor. The results showed that for both nontreated (Fig2C) and Enzalutamide treated tumors (Fig2E), the tumors derived from AR-low and -hi cells maintained their relative low and hi AR activities compared to each other. These results suggest that, opposite to our original hypothesis, prostate cancer with higher AR activity might be more resistant to AR-targeted therapy.

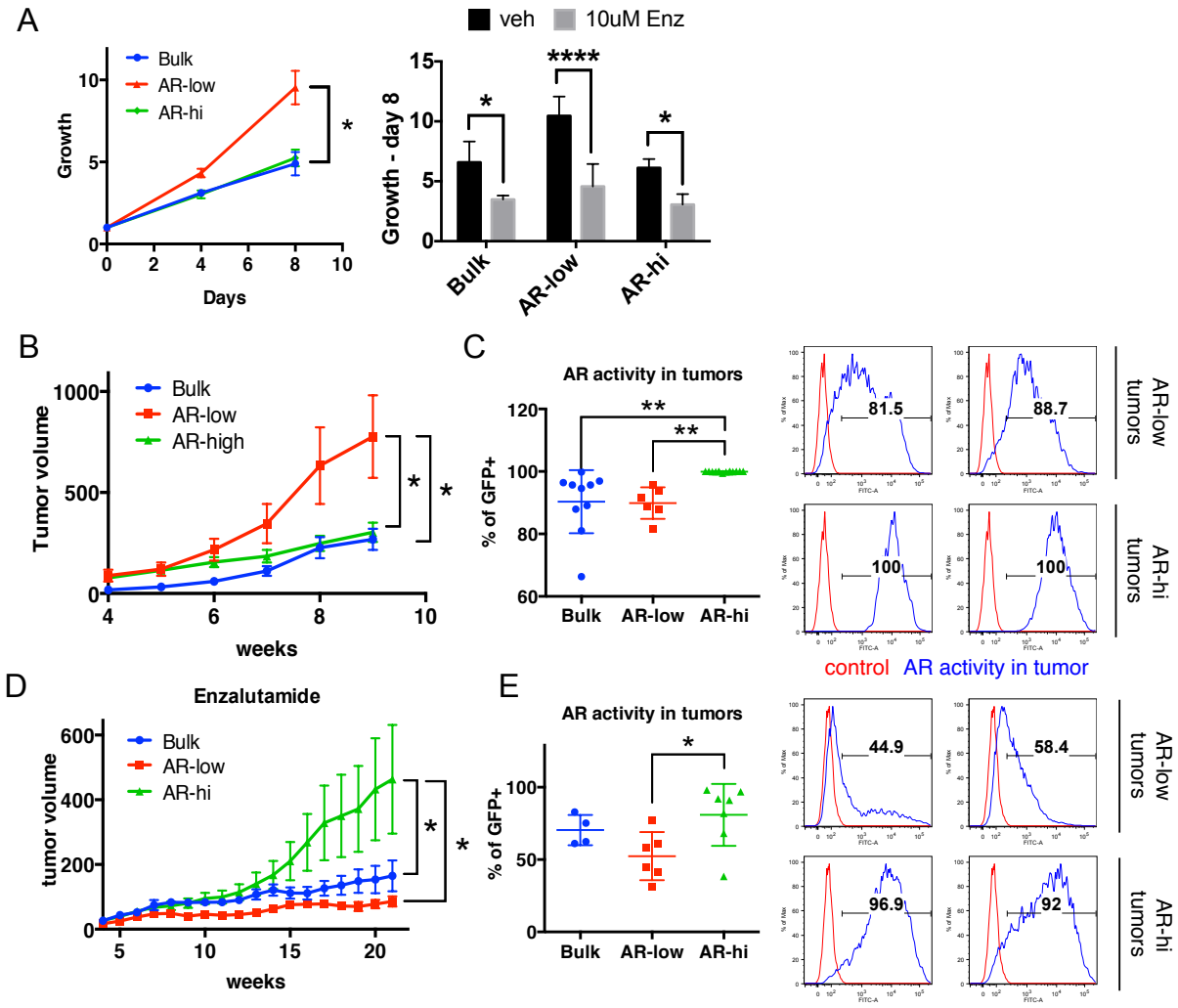

Figure 2. AR-hi prostate cancer cells are more resistant to Enzalutamide treatment. (A) AR-low cells grow faster than AR-hi cells and more sensitive to Enzalutamide. (B) Tumors derived from AR-low cells grow faster than tumors derived from other populations. (C) At the end the experiment described in (B), the percentage of the cells expressing GFP AR reporter were analyzed in individual tumor. The representative flow cytometry results are shown in the right panel (D) Tumors derived from AR-hi cells are more resistant to AR targeted therapy than other populations. The sorted AR-low and –hi cells were injected into castrated mice and treated with Enzalutamide immediately after injection. (E) At the end the experiment described in (D), the percentage of the cells expressing GFP AR reporter were analyzed in individual tumor. The representative flow cytometry results are shown in the right panel

Specific Aim 2: Identify factors responsible for higher AR activity in AR-hi cells

2-1. Small scale-shRNA screen with selected genes to find factors regulate AR-transcriptional activity in AR-hi cells.

Completion date: 03.2016-04.2016 (2 months)

Percentage of completion: 100%

Results: The RNA-sequencing was conducted to further characterize the molecular features of AR-low and AR-hi cells. The gene set enrichment analysis (GSEA) shows that the gene sets defined by genes up- and down-regulated by androgen were enriched in AR-hi and AR-low cells as expected, respectively (Fig3A). However, the RNA-sequencing data did not show any known AR-cofactors differentially expressed between AR-low and AR-hi cells. To identify factors that may be responsible for higher AR activity in AR-hi cells, we performed shRNA-mediated knockdown study with 47 selected genes up-regulated in AR-hi cells (Fig3B). The results showed that the knockdown of GREB1, GHRHR or KLF8 inhibited AR activity in AR-hi cells (Fig3C). We also found that each of these genes is regulated by AR (Fig3D), which explains their increased expression in AR-hi cells. Because GREB1 knockdown had the largest effect on AR activity, and based on its reported function as an ER cofactor (7), we selected GREB1 for more detailed characterization.

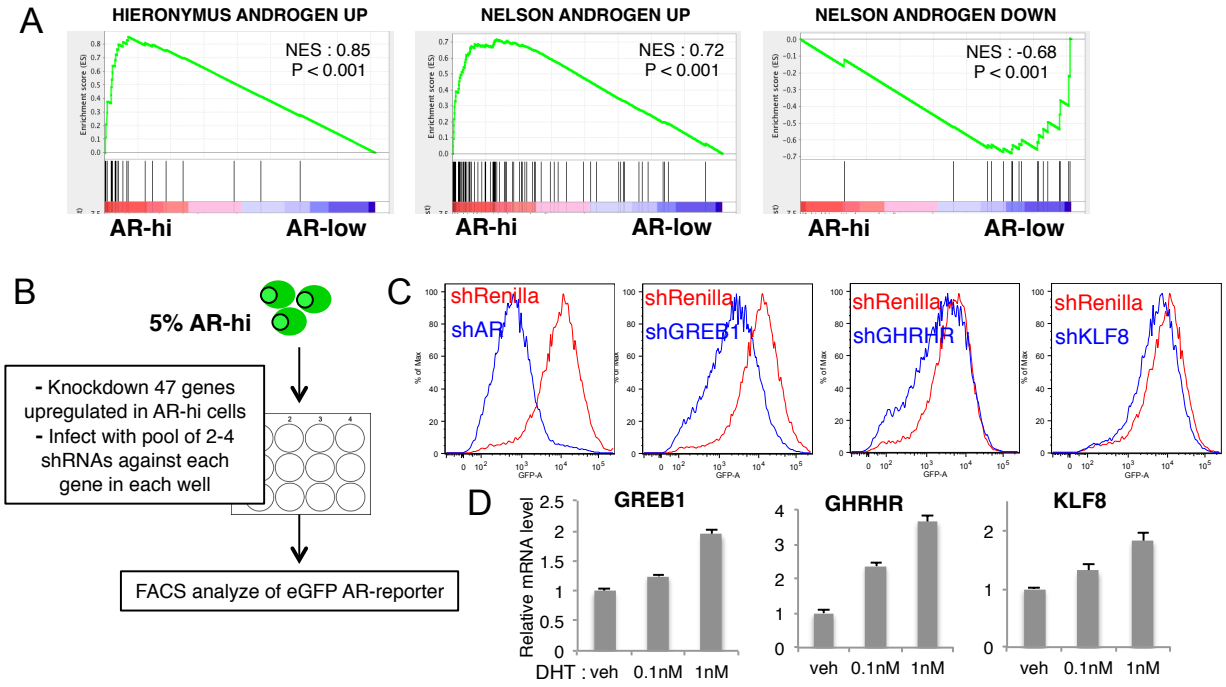

Figure 3. Small scale-shRNA screen with selected genes to find factors regulate AR-transcriptional activity in AR-hi cells. (A) GSEA profile shows that the gene sets defined by genes up-regulated by androgen was enriched in AR-hi cells (2 left graphs) and genes down-regulated by androgen was enriched in AR-low cells (right graph). (B) The schematic of knockdown study with 47 selected genes up-regulated in AR-hi cells. (C) The flow cytometry results show that the knockdown of GREB1, GHRHR and KLF8 inhibited AR reporter activity. AR shRNA was used as a control. (D) The transcription of GREB1, GHRHR and KLF8 is regulated by androgen.

2-2. Test the effect of GREB1 knockdown on AR transcriptional activity.

Completion date: 05.2016-08.2016 (4 months)

Percentage of completion: 100%

Results: Knockdown of GREB1 inhibited AR target gene expression in both AR-low and –hi cells (Fig4A and B) and suppressed the enhanced DHT-induced AR target gene expression in AR-hi cells (Fig4C). These results suggest that GREB1 acts as a potential AR-cofactor in both AR-low and –hi populations and the increased level of GREB1 in AR-hi cells is one of the factors responsible for enhanced AR-activity in this population. To further investigate the role of GREB1 on AR transcriptional activity, AR-hi cells with control or GREB1 shRNA were subjected to RNA-sequencing. The heatmap generated from gene expression data shows that the gene set up-regulated by DHT (Androgen UP) was suppressed by GREB1 knockdown and the gene set down-regulated by DHT (Androgen DOWN) was activated in GREB1 knockdown samples (Fig4D). We then analyzed number of genes up-regulated by DHT in each group, which showed that 70.5% of DHT-induced genes in control cells were inhibited by GREB1 depletion (Fig4E). GREB1 is known to promote estrogen receptor (ER) activity by enhancing ER interaction with its cofactors, including the p300/CBP complex (7). Consistent with this, we saw that AR-hi cells showed increased p300 binding on AR target genes (Fig4F), which was dependent on GREB1 (Fig4G), suggesting that GREB1 also promotes AR transcriptional activity by recruiting p300 to AR. Interestingly, the GREB1 depleted AR-low and –hi cells also showed decreased AR binding on AR target gene (Fig4H), indicating that GREB1 might affect AR activity by modifying AR cistrome. We next tested the effect of GREB1 loss of function on cell growth. The CRISPR/Cas9-mediated inhibition of GREB1 function suppressed growth of AR-hi cells that are further inhibited by Enzalutamide treatment (Fig4I), showing the therapeutic potential of targeting GREB1.

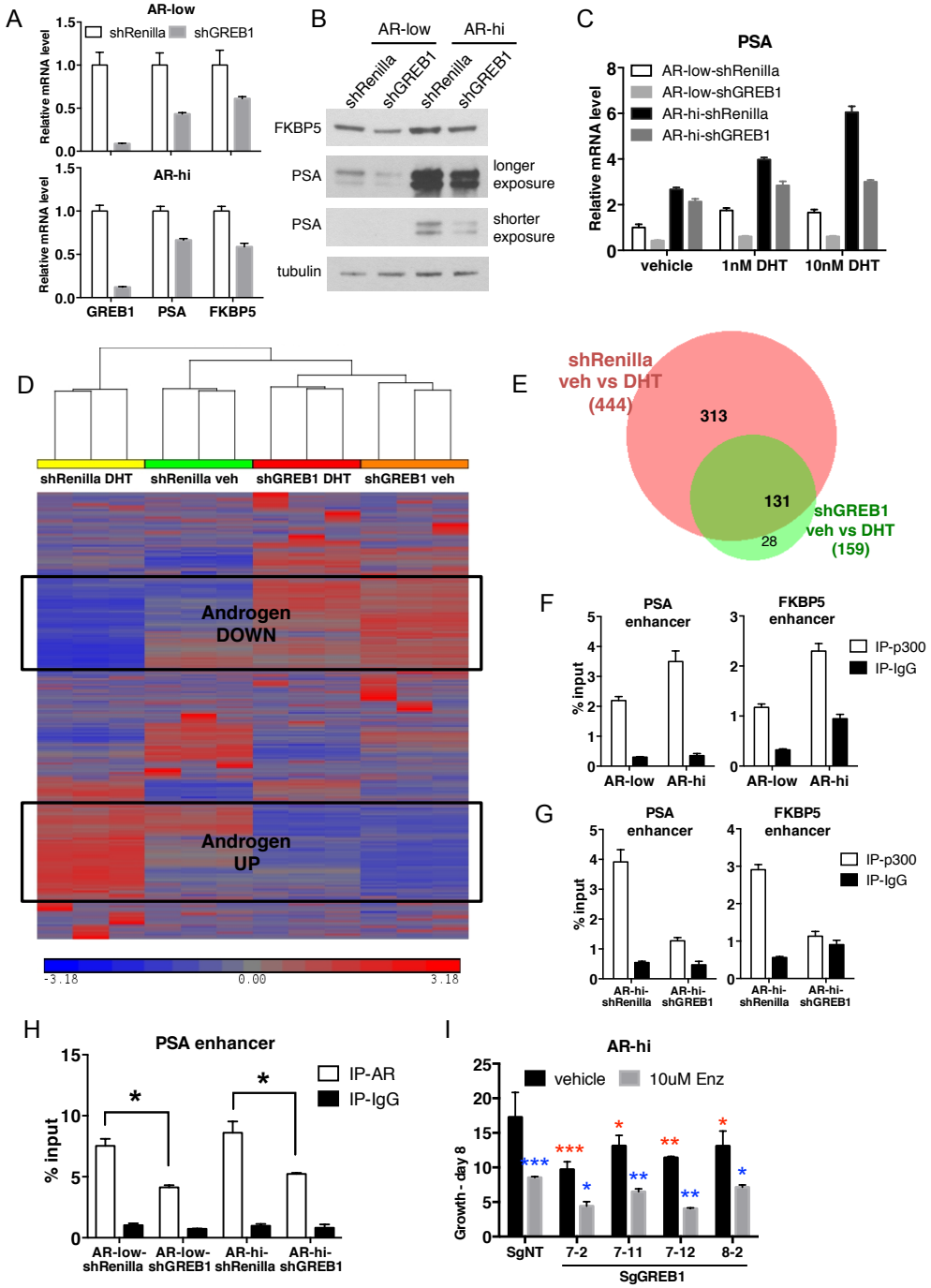

Figure 4. Knockdown of GREB1 suppresses AR transcriptional activity by inhibiting p300 and AR recruitment to target genes. (A-B) Knockdown of GREB1 inhibited AR target gene expression in both AR-low and -hi cells. (C) Knockdown of GREB1 suppressed the enhanced AR transcriptional activity in AR-hi cells. (D) The heatmap generated from gene expression data shows that the gene set up-regulated by DHT (Androgen UP) was suppressed, and the gene set down-regulated by DHT (Androgen DOWN) was activated by GREB1 knockdown in AR-hi cells. (E) The venn diagram showing that 70.5% of DHT-induced genes in control AR-hi cells was inhibited by GREB1 knockdown. (F-G) AR-hi cells have increased p300 binding on AR target genes in a GREB1 dependent manner. (H) GREB1 knockdown inhibits AR recruitment to PSA enhancer. (I) The CRISPR/Cas9-mediated inhibition of GREB1 function suppresses growth of AR-hi cells. * control (SgNT) vs. each GREB1 inhibited lines (SgGREB1); * vehicle vs. Enzalutamide treatment in each line.

Opportunities for training and professional development:

The training goal for my post-doctoral fellowship is to strengthen my scientific independence by improving critical research thinking and experimental techniques. To do so, during the first award period, I attended internal and external meetings/conferences. In addition to the weekly lab meetings, I attended MSKCC seminar series featuring speakers in cancer biology and translational research. I also attended the annual American Association for Cancer Research (AACR) meeting where I had opportunity to share scientific ideas with eminent researchers across the world. During this first training period, I learned technical skills related to the xenograft assay and RNA-sequencing and also learned how to analyze the data I obtained from these studies. As I continue with this work, I'm also learning how to validate the candidate molecular factors that might have clinical significance in disease progression by interacting with physicians in the Sawyers laboratory and throughout MSKCC.

Results disseminated to communities of interest:

Nothing to report

Plan for the next reporting period:

Our data from the first training period provided evidence that prostate cancer cells with varying AR activities have different molecular characteristics. We also showed that the tumors with higher AR activity were more resistant to AR-targeted therapy, and inhibition of GREB1 function in this population suppressed the cell growth, highlighting the importance of studying GREB1 in prostate cancer. GREB1 was reported as an ER interacting protein in breast cancer. Based on functional and structural similarity between ER and AR, we will test if GREB1 interacts with AR and binds to AR binding sites on DNA. We will also test if overexpression of GREB1 promotes growth and AR transcriptional activity in prostate cancer cells. To further understand the role of GREB1 in AR cistrome, we will conduct AR ChIP-sequencing in GREB1 depleted cells to see if AR peaks and distribution is modified by GREB1 knockdown. In addition, we will also perform time-course ChIP experiment against AR, p300 and histone marks on control and GREB1 depleted cells, to study the impact of GREB1 knockdown on the dynamics of AR target gene regulation (8).

Impact

Impact on the development of the principal disciplines of the project:

Prostate cancer is the most common form of cancer in men. Given the key role of androgen receptor (AR) signaling in disease progression, the current approach to treat prostate cancer is AR-targeted therapy. While this initially results in tumor regression, aggressive disease typically recurs, making the treatment of what is now called castration-resistant prostate cancer (CRPC) the major challenge in the field (1). Charles Sawyers' laboratory developed a second-generation AR inhibitor, Enzalutamide (9), that has increased both patient survival and quality of life (10). However, resistance remains as a major problem with complex molecular mechanisms (2, 3), reflecting the heterogeneity of the disease. Increasing number of studies have shown the molecular and genetic diversity among prostate cancers (4, 5) including a wide range of AR activity (6). Given that AR is the central therapeutic target in this disease, this observation lead to an important question if tumors with different AR activity have differential response to AR-targeted therapy. Indeed, one of the resistance mechanisms to Enzalutamide is that a small subset of resistant tumors shows low or absent AR expression (11), which can explain the nonresponsiveness of tumors to AR inhibition. Given this, we hypothesized that the prostate cancer with low AR activity will be more sensitive to Enzalutamide treatment. Our data from the first training period suggests that the results are in fact the opposite, showing that the tumors with higher AR activity is more resistant to Enzalutamide treatment. We also identified GREB1 as a potential new AR cofactor that enhances AR activity and Enzalutamide resistance in tumors with higher AR activity.

The results of this work will provide valuable information on developing therapeutic strategies for prostate cancer patients. Given that GREB1 was upregulated in tumors with higher AR activity, GREB1 can be used as a marker to screen prostate cancer with high AR activity (high GREB1) that might not be benefitted from Enzalutamide treatment. Furthermore, our data showed that inhibition of GREB1 function in these tumors with high AR activity suppressed the cell growth, indicating the therapeutic potential of targeting GREB1. Therefore, further understanding of the function of GREB1 in prostate cancer will provide novel insights into the development of effective therapeutic approaches, which will ultimately decrease suffering and improve survival of prostate cancer patients.

Impact on the other disciplines:

Nothing to report

Impact on technology transfer:

Nothing to report

Impact on society beyond science and technology:

Nothing to report

Changes/Problems

Changes in approach and reasons for change:

In our initial proposal, we hypothesized that the AR-low prostate cancer cells might be more resistant to Enzalutamide treatment and proposed to further study this cell population. Our data form the first training period showed the opposite result that AR-hi cells are in fact more resistant to this treatment (Fig2 A and D). We also proposed to study the Enzalutamide resistant AR-low tumors that are derived from AR-hi cells, based on the hypothesis that AR-hi cells might be reprogrammed to AR-low status under AR-targeted therapy to become more resistant to this treatment. However, our data showed that the Enzalutamide resistant AR-high tumors maintained their higher AR activity compared to AR-low tumors (Fig2E). Therefore, we attempted to identify factors responsible for higher AR activity in AR-hi cells using knockdown study in combination with RNA-sequencing (Fig3). As a result, we found GREB1 as a potential new AR cofactor that is partly responsible for the higher AR activity and Enzalutamide resistance in AR-hi tumors (Fig4). Based on these results, we will further investigate the function of GREB1 in prostate cancer in the next training period.

Actual or anticipated problems or delays and actions or plans to resolve them:

Nothing to report

Changes that had a significant impact on expenditures:

Nothing to report

Changes in use or care of human subjects, vertebrate animals, biohazards, and select agents:

Nothing to report

Product

Publications, conference papers, and presentations:

Nothing to report

Website(s) or other Internet site(s):

Nothing to report

Technologies or techniques:

Nothing to report

Inventions, patent applications, and/or licenses:

Nothing to report

Other Products:

Nothing to report

Participants & other collaborating organizations

Individuals worked on the project:

Name: Eugine Lee
Project Role: PI
Nearest person month worked: 12
Contribution to Project: Dr. Lee has designed and performed all of the experiments during the periods.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

Special reporting requirements

None.

Appendices

References:

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